Annual Report - 2012

Prepared for the California Pear Board

Project Title:	Evaluation of new bactericides for control of fire blight of pears caused by *Erwinia amylovora*
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SUMMARY

1. Twenty-one isolates from eight pear orchards in Lake, Sutter, and Yuba Co. were obtained in surveys on antibiotic resistance in populations of *E. amylovora.* Isolates from Lake Co. were all sensitive to streptomycin, oxytetracycline, and kasugamycin. In one orchard each from Sutter and Yuba Co., a high level of streptomycin-resistance was present. All isolates of the pathogen belonged to the high-resistance category, and these isolates also showed reduced sensitivity to oxytetracycline. Thus reduced sensitivity to oxytetracycline was found in two additional orchards from the ones previously identified.
2. Two air-blast field trials were conducted on the management of fire blight. The following products were evaluated: the antibiotics kasugamycin (Kasumin), streptomycin (Firewall), and oxytetracycline (Fireline); copper hydroxide/copper oxychloride (Badge X2); the fungicide Quintec; the fermentation product polyoxin-D (Ph-D); the biocontrols *Streptomyces lydicus* (Actinovate) and *Aureobasidium pullulans* (Blossom Protect); the natural products Proalexin and Citrox, as well as the oxidizing sanitizer AgriTitan.
    a. The natural incidence of blight was low in 2012 (2.3-4% natural disease incidence in the controls). Still, kasugamycin continued to be highly effective in reducing the disease. Kasugamycin was also very effective in mixtures with streptomycin, oxytetracycline, polyoxin-D, or Actinovate.
    b. Badge also significantly reduced the incidence of disease and Blossom Protect was similarly effective to Kasumin in one study.
3. Kasugamycin (Kasumin) registration in the United States is being pursued on pome fruit with a California registration expected in late 2012.
4. In studies on the molecular mechanism of streptomycin resistance in *E. amylovora,* a new mode of resistance for moderately resistant isolates was confirmed where *strA-strB* resistance genes on transposon Tn*5393* are located on plasmid pEU30. This plasmid is also present in highly resistant isolates, but here it does not carry *strA-strB* genes. For these strains, resistance is due to a point mutation in a chromosomal gene.

INTRODUCTION

Fire blight, caused by the bacterium *Erwinia amylovora,* is a very destructive disease of pome fruit trees worldwide, especially pears. In addition to cankers, the pathogen overwinters in flower buds, diseased fruit, small twigs, and branches left on the ground after pruning. In the spring, blossoms are infected through natural openings in nectaries and pistils. After infecting the blossoms, the bacteria grow into the peduncles and spurs. During warm and humid weather, ooze droplets consisting of new inoculum, are exuded from the peduncles. Young fruitlets often become infected, and they also turn black, dry, shrivel, but usually remain attached to the tree. The disease spreads rapidly. After invading blossoms, the bacterial pathogen can invade adjacent leaves through stomata, trichomes, hydathodes, and through wounds caused by hail or wind whipping. Succulent twigs, suckers, sprouts, and shoots are the next tissues infected. Secondary infections may occur throughout the growing season. Inoculum is spread by wind, rain, insects, birds, or by man, e.g. by means of contaminated pruning tools. Primary and secondary infections may develop into the branch. At this time the infection, if walled off, produces a canker or it penetrates further into the branch and then into the trunk. From here the bacteria may move into other branches

and finally the trunk. Trunk cankers will eventually girdle the tree and the whole tree will die. The disease can be very severe in some years, causing repeated infections during warm and wet weather.

Control measures. Fire blight is one of the most difficult diseases to manage. The infection period is long, and moreover, very few effective chemicals are available. Integrated programs that combine sanitation and orchard management with chemical and biological controls are the best approaches. If the disease is in its early stage and only a few twigs are blighted, it often can be eliminated by pruning. Thus, aggressive and regular scheduled pruning of diseased tissue is essential for keeping inoculum levels low in an orchard.

Current chemical control programs for fire blight control are based on protective schedules, because available compounds are contact treatments and are not systemic. Copper compounds have been used since the early 1900s, mostly in the form of copper sulfate plus lime (Bordeaux mixture). Control with copper compounds is only satisfactory when disease severity is low to moderate. On Bartlett (summer) pears, copper treatments are widely used only during dormant and bloom periods because phytotoxicity commonly occurs on fruit as russeting. New formulations of copper, however, allow for reduced rates based on the metallic copper equivalent (MCE) and thus, extended usage past the bloom period may provide an effective rotational treatment or mix-partner without causing russeting. The antibiotic streptomycin came into general commercial use during the late 1950s, followed by the less effective oxytetracycline (terramycin). Because of lack of alternative control materials, these antibiotics are still being widely used. In our antibiotic resistance surveys, we confirmed the high incidence of streptomycin resistance in some of the main California pear growing regions. We also detected isolates of *E. amylovora* with reduced sensitivity to oxytetracycline at several locations over the years. At one of these locations field treatments with Mycoshield were reported to be ineffective in controlling the disease. In inoculation experiments using these strains we demonstrated that the disease could not be effectively controlled using oxytetracycline. Thus, field resistance has occurred in some locations.

New, more effective materials for fire blight control with a different mode of action from currently used bactericides have to be developed to combat this destructive disease. These could then be incorporated into a resistance management program where compounds are rotated and mixed. The most effective alternative treatment that we identified during the past years with an efficacy equal to streptomycin and oxytetracycline is the antibiotic kasugamycin (Kasumin). This compound has also shown very good efficacy in controlling fire blight in field trials in other pome fruit growing areas of the country. Although concerns have been expressed by regulatory agencies regarding the use of antibiotics in agriculture that are also used in human medicine, kasugamycin is not used in human and animal medicine and has a different mode of action from streptomycin or oxytetracycline (no cross-resistance). Through our efforts, registration of Kasumin in California is expected in late 2012.

Kasugamycin was again effectively used in our field trials in 2012. Kasumin was applied by itself and in mixtures with selected other materials, including biological treatments. This was done to identify effective mixture treatments that would reduce the potential for resistance development. A new material that we included in 2012 was AgriTitan, an oxidizing sanitizer for field use. Additionally, we continued to evaluate the biocontrols Actinovate (*Streptomyces lydicus*) and Blossom Protect (*Aureobasidium pullulans*), the natural products Citrox + ProAlexin, the fermentation product polyoxin-D (Ph-D), as well as the fungicide quinoxyfen (Quintec) that was shown to have antibacterial activity by us in the management of bacterial spot of tomato and by others for selected bacterial diseases. We also evaluated the reduced MCE copper compound Badge in a program with four consecutive sprays.

In another objective of our project we are investigating the molecular mechanism of streptomycin resistance in California isolates of *E. amylovora*. Several mechanisms have been described for isolates of the pathogen from various locations. The two major groups are: i) a point mutation in the chromosomal *rps*L gene; and ii) resistance genes *StrA* and *StrB* that are associated with a transposon (i.e., Tn*5393*) and that are most commonly located on one of several plasmids. Strains with a high level of streptomycin resistance are associated with the chromosomal gene; whereas, moderate streptomycin resistance is associated with the *StrA* and *StrB* genes in California. We have determined that the majority of recent streptomycin-resistant isolates in California have the *StrA* and *StrB* genes. These are, however, located on a plasmid that previously has not been found to carry resistance genes. This novel mode of resistance was further investigated in 2012 in an attempt to better understand the biology of the pathogen and how it responds to selection pressures.

OBJECTIVES

1. Evaluate and optimize the performance of the antibiotic kasugamycin (Kasumin) as compared to streptomycin, oxytetracycline (Mycoshield) in cooperation with UCCE.
   a. Laboratory in vitro tests to evaluate the bactericidal activity with and without pH buffering using spiral gradient dilution assays.
   b. Small-scale hand-sprayer tests using different treatment-inoculation schedules.
   c. Field trials with protective air-blast spray treatments at several locations: mix with buffering adjuvants to adjust the pH of spray solutions, fungicides (mancozeb, dodine), other antibiotics (streptomycin, oxytetracycline), new formulations of copper (e.g., Kocide 3000, Badge X2), and plant defense activators (e.g, ProAlexin, Citrox). Evaluate product rates, timings, and rotations.
   d. Evaluate the efficacy of integrated programs using copper, fungicides, antibiotics and biological controls (e.g., Actinovate, Blossom Protect) and natural products (e.g, ProAlexin, Citrox).
   e. Efficacy of sanitizing agents (Deccosan) and other treatments (titanium dioxide – AgriTitan)
2. Determine the distribution of streptomycin- or oxytetracycline -sensitive and -resistant strains of *E. amylovora* in pear orchards in California (continuation of 2006-11 surveys)
   a. Laboratory in vitro tests to evaluate the bactericidal activity with and without additives in amended agar assays.
   b. Characterization of streptomycin- and oxytetracycline-resistant strains using molecular approaches: characterize plasmids that harbor the resistance genes and compare to *E. amylovora* populations from other parts of the country.

MATERIALS AND METHODS

***Laboratory studies on the toxicity of bactericides against* E. amylovora.** Kasugamycin (Kasumin 2L, Arysta Life Sciences, Cary NC), streptomycin (Sigma, St. Louis, MO), and oxytetracycline (Sigma) were evaluated for their in vitro toxicity using the spiral gradient dilution method. For this, a radial bactericidal concentration gradient was established in nutrient agar media in Petri dishes by spirally plating out a stock concentration of each antimicrobial using a spiral plater (Autoplate 4000; Spiral Biotech, Inc., Norwood MA). After radially streaking out suspensions of the test bacteria (10 µl of 10^8 cfu/ml as determined by measurement of optical density at 600 nm) along the concentration gradient, plates were incubated for 2 days at 25ºC. Measurements were visually taken for two inhibitory concentrations: i) the lowest inhibitory concentration (LIC; the lowest concentration where inhibition of bacterial growth was observed, i.e., where the bacterial streak became less dense visually), and ii) the minimal concentration that inhibited growth by >95% (MIC). The actual antibiotic concentrations were obtained by entering the radial distances of inhibition (measured from the center of the plate) into the Spiral Gradient Endpoint computer program (Spiral Biotech, Inc.).

Isolation of* E. amylovora*, bacterial culturing, and verification of species identity. Pear samples with fire blight symptoms were obtained in the spring and early summer of 2012 from orchards in Yuba, Sutter, and Lake Co. Infected plant material (flowers, fruit, stems, and pedicels) was surface-disinfested for 1 min using 400 mg/L sodium hypochlorite, rinsed with sterile water, cut into small sections, and incubated in 1 ml of sterile water for 15 to 30 min to allow bacteria to stream out of the tissue. Suspensions were streaked onto yeast extract-dextrose-$CaCO_3$ agar (YDC). Single colonies were transferred and the identity of the isolates as *E. amylovora* was verified by colony morphology and by PCR using primers specific for the ubiquitous *E. amylovora* plasmid pEA29 described by Bereswill et al. (Appl. Environ. Microbiol. 58:3522-2536). The presence of a 1-kb DNA fragment after gel electrophoresis confirmed a positive identification. A total of 21isolates of *E. amylovora* from eight orchard locations were obtained in 2012.

***Field studies using protective treatments during the growing season*.** In two field studies in a commercial Bartlett orchard in Live Oak, four applications of selected treatments (see Results) were done on 3-26 (70% bloom), 4-2 (full bloom), 4-9 (petal fall), 4-14 (begin rattail), and 4-24-12 (rattail) using a back-pack airblast sprayer at 100 gal/A. One trial focused mostly on conventional treatments, whereas in the other trial biologicals (two biocontrols, two natural products), an oxidizing sanitizer (i.e., AgriTitan), and Kasumin were evaluated. Disease was evaluated in May 2012 and for this, the incidence of infected spurs of the total number of spurs evaluated was determined. Data were analyzed using analysis of variance and LSD mean separation procedures of SAS 9.1.

Characterization of streptomycin-resistant strains using molecular approaches. The location in the genome of the *strA-strB* resistance genes that previously were found to be associated with transposon Tn*5393* was characterized for representative California isolates of *E. amylovora*. Additionally, the exact location was determined on plasmid pEU30 by plasmid mapping. Plasmids were also isolated from sensitive, moderately resistant and highly resistant isolates using a commercial kit and digested with *Kpn*I. Fragments were separated on agarose gels and banding patterns were analyzed visually for the presence of pEU30. This work was done in collaboration with Dr. G. Sundin at Michigan State University.

RESULTS AND DISCUSSION

***Survey of antibiotic sensitivity among* E. amylovora *strains collected in California*.** Isolates of *E. amylovora* were confirmed for species identity by PCR amplification of a 1-kb DNA fragment using specific primers for plasmid pEa29 that is ubiquitously found in this bacterium. A total of only 21 isolates from 8 pear orchard locations in Sutter, Yuba, and Lake Co. (1 to 5 isolates per location) were obtained and tested for their sensitivity against streptomycin, oxytetracycline, and kasugamycin. Still, this limited survey produced some interesting results.

All isolates from Lake Co. were found to be sensitive against the three antibiotics (Table 1). In previous surveys, most isolates from the Northern growing regions were also sensitive, although high levels of resistance against streptomycin were present at locations in Sacramento Co. In 2012, a high level of streptomycin-resistance was present in two orchards, one in Sutter and one in Yuba Co. All isolates of the pathogen belonged to the high-resistance category, and minimum concentrations to completely inhibit growth of the bacterium exceeded 50 ppm. Furthermore, these resistant isolates all also had a reduced sensitivity to oxytetracycline. MICs of isolates sensitive to oxytetracycline were 0.09 - 0.38 ppm; whereas those for isolates with reduced sensitivity were 1.25 to 1.88 ppm. Thus, this is very similar to what we observed at a few orchard locations in 2007 and 2009: isolates with high resistance to streptomycin had a reduced sensitivity to oxytetracycline. In contrast, other isolates with high-streptomycin resistance that we found in our previous surveys were sensitive to oxytetracycline. High-streptomycin resistance that is due to a chromosomal mutation was the first type of streptomycin resistance described in West coast growing areas (Chio and Jones, 1995). Based on our surveys over the years, his type of resistance has been mostly replaced by moderate resistance where *strA-strB* resistance genes are located on plasmids. All isolates collected in 2012 were sensitive to kasugamycin.

***Field studies using protective treatments during the growing season*.** In the first trial on Bartlett pear, a range of treatments was evaluated: antibiotics (streptomycin, oxytetracycline, kasugamycin), copper (Badge X2), a fungicide (quinoxyfen – Quintec), and a bio-fungicide (polyoxin-D – Ph-D). The natural incidence of disease was low in the untreated control in this orchard with only 2.3% of the twigs showing an infection. Most treatments (except Ph-D that was not effective) numerically reduced the incidence of blight, but due to variability among the four tree replicates, in some cases there was no significant difference to the control (Fig. 1). Five of the seven treatments where no disease was detected all contained Kasumin, either by itself or in a mixture with streptomycin, oxytetracycline, or Ph-D. The other two treatments with no disease were streptomycin by itself or mixed with oxytetracycline. Quintec and Badge only numerically reduced the incidence of disease as compared to the control. No phytotoxicity (russeting) on the developing Bartlett fruit was observed after four applications with any of the treatments.

In the second trial, where biologicals (two biocontrols, two natural products), an oxidizing sanitizer (i.e., AgriTitan), and Kasumin were evaluated the incidence in the control was 4%. The oxidizing sanitizer AgriTitan was not effective (Fig. 2) and higher rates will need to be evaluated. No disease was detected using the biocontrol Blossom Protect by itself , but when mixed with Actinovate or with Citrox and ProAlexin its efficacy was reduced, indicating a negative interaction. Over the last two years with low to intermediate disease pressure, Actinovate and Blossom Protect showed consistent efficacy in reducing the incidence of fire blight, but efficacy was not as high as for some of the chemical treatments (e.g., streptomycin, kasugamycin). New treatments will be available for 2013 and these will be tested in comparative studies. In this latter trial, Kasumin by itself or mixed with Actinovate also resulted in no disease in this second plot. Thus, Actinovate had no negative effect on Kasumin. Kasumin however, was found to be inhibitory to *Streptomyces lydicus*, the biocontrol agent in Activovate, in in vitro bioassays. In

additional studies, Kasumin was also inhibitory to *Aureobasidium pullulans*, the biocontrol yeast in Blossom Protect. In contrast, *A. pullulans* was not affected by streptomycin or oxytetracycline; whereas, S. *lydicus* was inhibited by both antibiotics. These in vitro studies can help to design compatible mixture and rotation programs with antibiotics and biocontrols.

Thus, although disease pressure was very low in 2012, kasugamycin continued to be highly effective in reducing the incidence of fire blight. Once registered, it can be used in resistance management programs with rotations and possibly mixtures. Registration of the product for California is expected for late 2012.

Characterization of streptomycin-resistant strains using molecular approaches. High-resistance to streptomycin in isolates from our surveys was previously found to be correlated with a mutation in the ribosomal protein S12 (*rps*L) gene located on the bacterial chromosomal, similar as was described for West coast isolates by Chio and Jones in 1995. We continued to investigate the molecular mechanism of moderately streptomycin resistance that is based on acquisition of *strA-strB* resistance genes. Based on our surveys over the past seven years, this type of resistance is currently much more common than the high-resistance based on a chromosomal mutation. We previously had confirmed the presence of *strA-strB* and transposon Tn*5393*. It was found to be located on plasmid pEU30 that has been described from isolates from the western United States in 2004, and not on plasmid pEa34 or pEa29 as has been described from Michigan. Thus, California isolates show a unique mode of resistance. PCR amplifications confirmed the association of *strA-strB* with pEa30 in all evaluated moderately resistant isolates that were collected between 2006 and 2011 from various locations in California (no moderately resistant isolates were found in 2012 due to limited sampling). Based on restriction enzyme analysis, plasmid pEU30 is also present in highly resistant isolates (Fig. 3), but does not carry *strA-strB*. We continued to molecularly analyze this new mode of resistance, and in collaboration with G. Sundin, were able to determine the insertion site of the resistance genes in the plasmid. This information, together with our streptomycin resistance survey data, is currently being prepared for publication.

Table 1. Incidence of resistance against streptomycin, oxytetetracycline, or kasugamycin in isolates of *Erwinia amylovora* collected in surveys of 8 California pear orchards in 2012

Orchard No.*	County	No. of isolates	Incidence streptomycin resistance (%)**	Incidence reduced oxytetracycline sensitivity (%)***	Incidence kasugamycin resistance (%)****
1	Sutter	2	0	0	0
2	Sutter	5	80**	80	0
7	Sutter	3	0	0	0
3	Yuba	4	100**	100	0
4	Lake	1	0	0	0
5	Lake	2	0	0	0
6	Lake	1	0	0	0
8	Lake	3	0	0	0
Total		21			

* - Note that some orchards had several blocks

** - Inhibitory concentrations were determined on nutrient agar using the SGD method. Minimum inhibitory concentrations (MIC, >95% inhibition) of isolates sensitive to streptomycin were 1.0 - 2.7 ppm; whereas those of isolates resistant to streptomycin were >50 ppm.

***- MICs of isolates sensitive to oxytetracycline were 0.09 - 0.38 ppm; whereas those for isolates with reduced sensitivity were 1.25 to 1.88 ppm.

****-MICs for kasugamycin were 3.8 to 16.1 ppm.

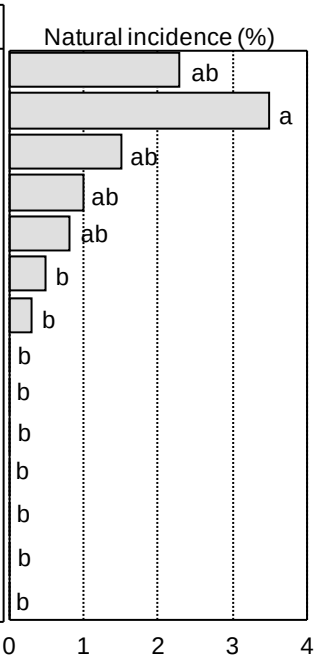
Table 2. Toxicity of antibiotics on in vitro growth of two biocontrol agents - *Streptomyces lydicus* and *Aureobasidium pullulans.*

	Toxicity against biocontrol agent*	
Antibiotic	*S. lydicus*	*A. pullulans*
Streptomycin	+	-
Oxytetracycline	+	-
Kasugamycin	+	+

* - Toxicity was determined using the spiral gradient dilution method. A "+" indicates that the antibiotic is toxic to the biocontrol organism at concentrations that are inhibitory to *E. amylovora* .

Fig. 1. Evaluation of new bactericides for fire blight management on Bartlett pears in a field trial in Live Oak CA - 2012

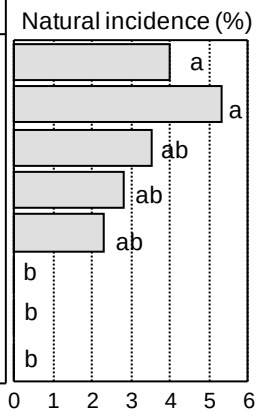
No.	Treatment*	Rate/A
1	Control	---
2	Ph-D	6.2 oz
3	Quintec	6 fl oz
4	Kasumin 8L + Quintec	100 ppm + 6 fl oz
5	Badge X2	8 oz
6	Fireline	200 ppm
7	Kasumin 8L + Badge X2	100 ppm + 8 oz
8	Kasumin 8L + Firewall	100 ppm + 100 ppm
9	Kasumin 8L + Fireline	100 ppm + 200 ppm
10	Kasumin 8L	100 ppm
11	Kasumin 2L	100 ppm
12	Kasumin 8L + Ph-D	100 ppm + 6.2 oz
13	Firewall	100 ppm
14	Fireline + Firewall	200 ppm + 100 ppm



Treatments were applied on 3-26 (70% bloom), 4-2 (full bloom), 4-9 (petal fall), 4-14 (begin rattail), and 4-24-12 (rattail) using an airblast sprayer at 100 gal/A. Disease was evaluated in May 2012.

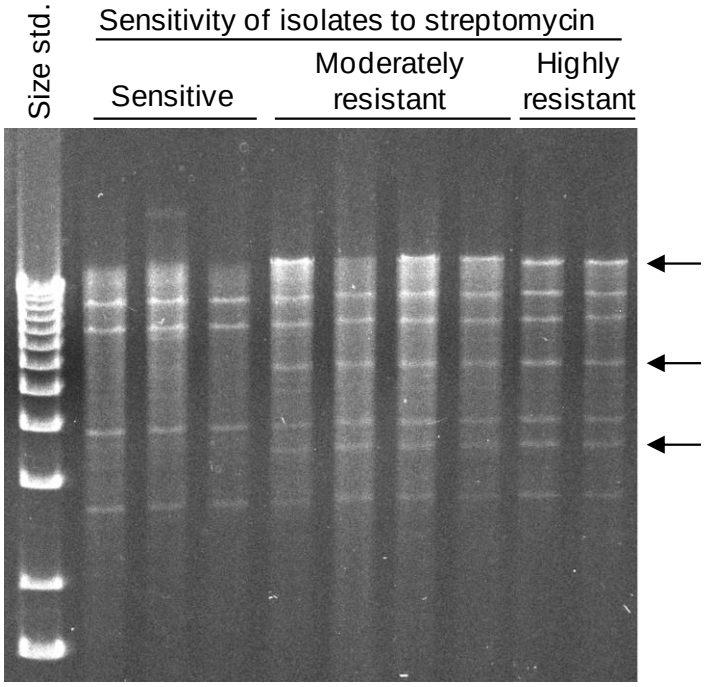
Fig. 2. Evaluation of new biologicals and antibiotics for fire blight management on Bartlett pears in a field trial in Live Oak CA - 2012

No.	Treatment	Rate/A
1	Control	---
2	AgriTitan	1:25
3	Blossom Protect+Buffer+Citrox+ProAlexin	1.34 lb+9.35 lb+133 ml+133 ml
4	Blossom Protect + Buffer + Actinovate	1.34 lb + 9.35 lb + 12 oz
5	Actinovate	12 oz
6	Kasumin 8L	100 ppm
7	Kasumin 8L + Actinovate	100 ppm + 12 oz
8	Blossom Protect + Buffer	1.34 lb + 9.35 lb



Treatments were applied on 3-26 (70% bloom), 4-2 (full bloom), 4-9 (petal fall), 4-14 (begin rattail), and 4-24-12 (rattail) using an airblast sprayer at 100 gal/A. Disease was evaluated in May 2012.

Fig. 3. Presence of plasmid pEU30 in isolates of *E. amylovora* with different levels of sensitivity against streptomycin



Plasmids were isolated using a commercial kit and digested with *Kpn*I. Arrows indicate restriction fragments of plasmid pEU30 that are present in moderately and highly resistant isolates, but not in sensitive isolates. Only moderately resistant isolates carry *strA-strB* genes on pEU30.